The effect of sex hormone-binding globulin (SHBG) protein polymorphism on the levels of SHBG, testosterone, and insulin in healthy Indonesian men

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Background: Sex hormone-binding globulin (SHBG) is a protein. The synthesis of SHBG protein is encoded by SHBG gene. Protein polymorphism of SHBG can occur because of a mutation of D327N exon 8 in SHBG gene. D327N mutation causes the addition of glycosylation so that molecular weight of SHBG protein increases. Protein polymorphism of SHBG is suspected to affect the levels of SHBG, testosterone, and insulin in the blood circulation.

Objective: To determine the effect of protein polymorphism of SHBG on the levels of SHBG, testosterone, and insulin in healthy adult men.

Materials and Methods: Serum samples were collected from 179 healthy adult men. Electrophoresis and western blotting were performed to determine the phenotype of SHBG. All subjects were divided into two groups based on SHBG phenotype; group I (normal SHBG phenotype) n = 159 subjects and group II (variant SHBG phenotype) n = 38 subjects.

Result: The levels of serum SHBG, testosterone, and insulin were measured using radioimmunoassay method. The levels of SHBG, total testosterone, and free testosterone between normal SHBG phenotype do not differ with variant SHBG phenotype (p > 0.05), but insulin levels between normal SHBG phenotype compared with variant SHBG phenotype was different (p < 0.05).

Conclusion: Protein polymorphisms of SHBG was not found to affect the levels of SHBG, total testosterone, and free testosterone. Protein polymorphism of SHBG was found to affect the insulin levels in the blood serum.

KEY WORDS: D327N mutation, insulin, protein polymorphism, sex hormone-binding globulin, testosterone

Abstract

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Introduction

Sex hormone-binding globulin (SHBG) is protein molecule in the circulation system. The largest component of SHBG is an amino acid molecule. Amino acid component of SHBG molecule is obtained as a result of SHBG gene expression in hepatocytes. After the SHBG molecule is synthesized in hepatocytes, it is secreted into circulation system, which
transports sex steroids in the blood[3] and regulates the availability of these hormones on target cells.[4]

SHBG gene is composed of 4.3 kilobases (kbs).[5] found in chromosome 17 p 3.1. Eight exon-long transcript whose expression to encode SHBG molecule is regulated by a downstream promoter.[6] Exon 1 is coding sequence for secretion of polypeptide signal, while exons 2–8 encode two parts of laminin G-like (LG) domains.[3] The amino acid-terminal of LG domains encoded by exons 2–4 consist of steroid binding and the part for dimerization,[4] and cation binding.[7] The carboxy-terminal LG-domain consists of two N-glycosylation (NG) sites; however, their biological importance continues to be obscure.[4]

Human SHBG molecule contains 373 amino acids (40.59 kDa) and 29 amino acids as for peptide signal.[8] In the circulation system, each molecule of SHBG acts as a homodimer subunit. Two molecular subunits of SHBG interact to form dimers.[8] Studies of the interactions between SHBG and synthetic steroids[9] also led to the conclusion that the molecular structure that best fits the SHBG steroid-binding site is the biologically active androgen, 5α-dihydrotestosterone (DHT).[4] The understanding that the steroid-binding and dimerization domains of human SHBG are situated within its amino-terminal LG-like domain urged us to crystalize this region of the molecule in a complex with DHT.[11]

SHBG molecule heavily glycosylated is the process of protein modification by adding or binding of sugars such as oligosaccharide. There are two forms of human SHBG glycosylation, which is N-glycosylation (NG) and O-glycosylation (OG). Human SHBG molecule contains NG at two places, which are asparagin (asn) 351 and 367, while OG at only one place, which is threonine 7 (Thr 7).[12] Addition of NG can happen to SHBG molecule, thereby increasing the molecular weight and result in protein polymorphism of SHBG. Protein polymorphism of SHBG can occur among others owing to a point mutation in exon 8. The missense mutation in exon 8 (GAC→AAC) causing the amino acid exchange aspartat (asp) to asn in codon 327 (D327N).[13] There are three types of SHBG phenotype, that is, normal SHBG (wild-type SHBG = W/W), heterozygous variant SHBG (W/\nu), and homozygous variant SHBG (\nu/\nu). Normal SHBG subunit consists of light subunit (L subunit) of 48 kDa and heavy subunit (H subunit) of 52 kDa. SHBG both homozygous and heterozygous variants have a superheavy subunit (SH subunit) of 56 kDa.[13]

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB) can be used to distinguish the molecular phenotype of normal and variant SHBG. Normal SHBG phenotype shows two bands (double banded), whereas the variant SHBG phenotype (homozygous and heterozygous variant SHBG) shows three bands (triple banded). The third band has a different molecular weight.[13]

Result of the research shows that additional carbohydrate chain at SHBG molecule shows no effect on the steroid-binding activity of SHBG, but it may reduce the plasma clearance of SHBG and account for a modest accumulation of the variant protein in the blood. It has been proven that point mutation in exon 8 causing SHBG genetic polymorphisms influences production and metabolism of SHBG, thus causing variation of SHBG levels between individuals.[14] Besides that, point mutation in exon 8 increases SHBG levels.[13]

Many factors that affect the levels of SHBG plasma include gender,[16] age,[17] hormonal and nutrition status,[18] heredity, and pathological conditions.[19,20] Furthermore, supplementation of isoflavones for 6 months decreases SHBG levels of postmenopausal women with osteopenia and osteoporosis.[21] Therefore, the objective of this study was to analyze the influence of SHBG protein polymorphism on SHBG, testosterone, and insulin levels in healthy adult men.

Materials and Methods

Research Design

This is a cross-sectional study with two hundred and twenty-three subjects assessed for eligibility.

Ethical Clearance

This study was approved by the Commission on Research Ethics, Faculty of Medicine, University of Trisakti, Jakarta, Indonesia. For participating in this study, all subjects gave written informed consent.

Sample Size

The number of subjects in each of the two groups was calculated. The minimum sample size was 14 subjects per group.

Study Subjects

One hundred ninety-seven subjects residing at Jakarta, who fulfilled the inclusion criteria were divided into two groups based on SHBG phenotyping. Group I: normal SHBG phenotype; group II: variant SHBG phenotype. Inclusion criteria in this study were healthy men represented by physical examination by doctor, aged 31–60 years. Exclusion criteria included conditions that affect the production of SHBG, including: cirrhosis of the liver; gonadal abnormalities; and medications such as phenytoin, androgens, growth hormone, high-dose glucocorticoids, diazoxide, epinephrine, cortisol, progesterin, and sulfonylureas. Subjects who were not present during the retrieval of research data were excluded. Besides that, a subject who had a blood sample lysis was excluded.

Serum Analysis

Fasting blood was collected from vena cubiti for all subjects. Fasting blood collection was done in the morning. Serum from 10 milliliters venous blood was isolated by centrifugation at 3,000 rpm for 20 min. Serum was stored at −20°C. SHBG phenotyping was carried out at the Laboratory of Biology, Faculty of Medicine, University of Indonesia, while the measurement of SHBG, total testosterone (TT), and free testosterone (FT) were carried out at the Laboratory of Endocrinology, Faculty of Medicine, University of Indonesia.
SHBG Phenotyping

Determination of SHBG phenotype was performed using SDS-PAGE and WB. SHBG in the serum sample has a molecular weight of about 100 kDa. Before electrophoresis, albumin in the serum was eliminated with Blue Sepharose. SHBG in the serum sample experienced a separation based on molecular weight when SDS-PAGE was done. SDS binding along the polypeptide chain and complex protein—SDS proportional to molecular weight also occurred. Band of SHBG that resulted in the gel separation was then transferred to a nitrocellulose membrane (immobilon). Band of SHBG on immobilized reacted with polyclonal rabbit anti-human SHBG immunoglobulin (A350) (Dako Denmark A/S, Glostrup, Denmark). After the bands of SHBG reacted with polyclonal rabbit anti-human SHBG immunoglobulin, they then reacted with polyclonal goat anti-rabbit anti-human SHBG immunoglobulin (D487) (Dako Denmark A/S, Glostrup, Denmark) and further reacted with alkaline phosphatase. After that, the membrane was washed with Nonidet P-40 in tris-buffered saline (TBS). Then it was stained with 5-bromo-4-chloro-3-indolyl-phosphatase and nitroblue tetrazolium (NBT) to see the bands of SHBG. Bands of serum SHBG samples were compared with band of standard SHBG (control). The band number of serum SHBG samples were used to determine SHBG phenotype, which was normal SHBG phenotype (two bands of SHBG = double-banded SHBG) and variant SHBG phenotype (three bands of SHBG = triple-banded SHBG).

Measurement of SHBG, Testosterone, and Insulin

The SHBG, TT, and FT levels were measured by radioimmunoassay (RIA) using commercial IRMA-Count SHBG [Diagnostic Products Corporation (DPC), Pacific Concourse Drive, Los Angeles, California, USA]. The TT levels were measured by RIA using commercial Coat-A-Count TT (DPC, Pacific Concourse Drive, Los Angeles, California, USA). The FT levels were measured by RIA using commercial Coat-A-Count FT (DPC, Pacific Concourse Drive, Los Angeles, California, USA). The insulin levels were measured by RIA using commercial Coat-A-Count insulin (DPC, Pacific Concourse Drive, Los Angeles, California, USA).

Analysis of all sera was done once in duplicate. Before the measurement of hormone levels, standardization of measurement was performed, which included sensitivity and interassay coefficients of variation (CVs).

Statistical Analysis

The levels of SHBG, TT, FT, and insulin were compared between groups by independent sample t-test.

Result

Research diagram to obtain the subjects is shown in Figure 1. Table 1 shows the characteristics of subjects.

WB analysis of SHBG protein shows normal SHBG and variant SHBG phenotype. Estimated molecular weight of low molecular weight (LMW) and standard of SHBG and serum SHBG in the samples are presented in Table 2.

Molecular weight of SHBG standard expected 45.7 kDa of L subunits and 51.7 kDa for H subunits. Molecular weight of normal SHBG phenotype in the sample showed two bands, predicted to be 46.4 kDa of L subunits and 52.1 kDa for H subunits. Molecular weight of variant SHBG phenotype in the sample showed three bands, predicted to be 46.4 kDa of L subunits, 52.1 kDa for H subunits, and 56.2 kDa for very heavy subunit. WB analysis of SHBG protein is presented in Figure 2.

Comparison of SHBG, testosterone, and insulin levels between normal SHBG and variant SHBG phenotype groups is presented in Table 3.

Discussion

In this study, molecular weight estimation of SHBG protein shows the variation with the results of research conducted by researchers previously.[14,15,22]

Results of research showed that normal SHBG phenotype consists of two subunits with different sizes, namely 49 and 52 kDa in the ratio 1:10, while variant SHBG phenotype shows three bands (triple banded) that consist of 49, 52, and 56 kDa.[12] According to research results by Cousin et al.,[14] the variant SHBG molecule consists of 48, 52, and 56 kDa.[14]

Difference in the molecular weight estimate of SHBG protein in this study with researchers earlier might be owing to electrophoresis conditions that vary in each treatment. Electrophoresis conditions are not the same in each treatment mainly because of condition of gel, running buffer solution, and TBS. Variations occur despite electrophoresis conditions have been created based on their size. The assumption has reinforced reports that gel electrophoresis is known for its often unsatisfactory precision. The percentage of relative standard deviations (RSD%) was in a range of 15%–70%.[29]

The results of electrophoresis and WB show the SHBG bands and bands of another proteins with molecular weight greater than SHBG molecular weight. This is probably owing to polyclonal antibody used that reacts with a molecule of SHBG and other protein molecules in the serum. Moreover, it may also be caused owing to SHBG molecule in the serum to form aggregates during storage. The aggregate formation that causes the denaturation process is not perfect; so, it appears as some protein bands. The generation of cross-reactive antibodies depends on the conformational stability and integrity of the immunogen and on the molecular form of its application, that is, free, polymerized, or carrier-bound.[24]

In this study, 197 healthy adult men were included. The number of individuals who showed a normal SHBG phenotype were as many as 159 (80.71%) and variant SHBG phenotype were 38 (19.29%). These data indicate differences in the frequency of SHBG phenotype distribution in Indonesia and some other countries. The study of 31 Swedish men showed 71% normal SHBG and 29% variant SHBG, whereas the study of 66 Swedish women showed 83% normal SHBG and 17% variant SHBG.[15]
Results of research in Belgium showed that 227 male and 174 female subjects showed 76% of normal SHBG phenotype and 24% of variant SHBG, while 268 subjects in Finland showed 84.3% normal SHBG phenotype and 15.7% of variant SHBG. Similar studies were also conducted by Van Baelen et al.[20] in Saudi Arabia, Zaire, China, and Korea. Results of these studies showed that the 151 men in Saudi Arabia showed 94% of normal SHBG phenotype and 6% variant SHBG. Studies on 203 subjects in Zaire showed 94% of normal SHBG phenotype and 6% variant SHBG, while 46 subjects in China showed 72% of normal SHBG phenotype and 28% variant SHBG. Result of research in Korea on 48 subjects showed 72.9% normal SHBG phenotype and 27.1% variant SHBG phenotype. Cousin et al.[14] in their study, have shown that, in men, the half-life value of homozygous variant SHBG genotype (W/W) or with higher SHBG levels 36.9 ± 15.9 nmol/L for W/v (n = 52) and 43.5 ± 3.5 nmol/L for v/v (n = 2) than was the wild-type W allele 31.1 ± 16.1 nmol/L (n = 249) (p = 0.039).[27] Consistently, the variant allele has been associated with increased SHBG levels in most studies,[19,27] but not all.[16,28]

Another study showed that there is no relation between D327N genotype and SHBG levels. Moreover, no association was found between serum SHBG concentration and the D327N genotype for either Black men or White men. They also showed the highest SHBG concentrations among men who were homozygous for the (TAAAA)6 allele but no difference in SHBG level according to D327N genotype. The absence of an association between SHBG level and D327N genotype in men contrasts with studies in women, suggesting a potential gender difference in the physiologic effect of the N327 allele.[19] On the basis of the abovementioned results, there is no difference in the levels of SHBG between the variant SHBG genotype when compared with normal SHBG genotype,[16] but another research has shown that the levels of SHBG in heterozygous variant SHBG (W/v) or homozygous variant SHBG (v/v) was higher than normal SHBG genotype group (W allele = WW).[27] Sensitivity of TT assay was 0.03 nmol/L. The interassay CVs were of 7.8% and 6.9% at TT concentrations of 2 nmol/L and 50.6 nmol/L, while the intraassay CVs were of 3.8% and 2.9% at the same TT concentrations, respectively. According to guidelines by Vankrieken[25] that TT levels in normal men were 6.93–28.1 nmol/L.[25] On the basis of these guidelines, TT levels for all subjects in this study are included in the range of normal values [Table 3]. Results of this study showed that TT levels between normal SHBG phenotype when compared with variant SHBG phenotype did not differ (p = 0.332).

There is a research that shows the relationship between D327N polymorphisms in SHBG and testosterone levels. The results of a study on 73 control women and 247 polycystic ovary syndrome (PCOS) showed that the variant allele carriers showed significantly lower levels of testosterone (p < 0.05) and 17-hydroxyprogesterone (p < 0.05), but the significance was achieved only in the age group older than 30 years. Thus, D327N polymorphism could influence the androgen levels.[29] Another study reported that low TT levels are associated with an increased risk of metabolic syndrome in men.[30,31] Moreover, circulating testosterone levels also have been associated with individual components of metabolic syndrome, such as insulin resistance.[30] Circulating testosterone is partly bound to SHBG with high affinity, and testosterone levels are strongly related to SHBG concentrations. SHBG levels also have been associated with the presence of genetic polymorphisms in SHBG affect the production and metabolism of SHBG, thus causing variation in SHBG levels between individuals. Other studies have shown that some genetic variants of SHBG are associated with circulating levels of SHBG and sex hormones.[26]

The results stated that the plasma concentration of SHBG was related to D327N allele v. The V allele was associated with higher SHBG levels 36.9 ± 15.9 nmol/L for W/v (n = 52) and 43.5 ± 3.5 nmol/L for v/v (n = 2) than was the wild-type W allele 31.1 ± 16.1 nmol/L (n = 249) (p = 0.039).[27] Consistently, the variant allele has been associated with increased SHBG levels in most studies,[19,27] but not all.[16,28]
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with the risk of metabolic syndrome in men.\textsuperscript{,30,31} SHBG has been reported to be associated with incident diabetes.\textsuperscript{19} Polymorphisms of the SHBG gene also have been linked with diabetes.\textsuperscript{19,33} Another result showed that SHBG levels importance as independent predictor of metabolic disorders, such as diabetes and metabolic syndrome.\textsuperscript{20} Futhermore, monosaccharide-induced lipogenesis reduces hepatocyte nuclear factor-4-alpha (HNF4A) levels, which in turn attenuates SHBG expression, and it can be noted that this provides a biologic explanation for the sensitivity of SHBG as a biomarker of the metabolic syndrome.\textsuperscript{24} In pathological conditions, a positive correlation was observed between testosterone levels and insulin sensitivity ($r = 0.4$, $p < 0.005$) and between SHBG and insulin sensitivity ($r = 0.44$, $p < 0.005$). Testosterone levels also correlated with $V_{O2max}$ ($r = 0.43$, $p < 0.05$) and oxidative phosphorylation gene expression ($r = 0.57$, $p < 0.0001$). Futhermore, this study showed that low serum testosterone levels are associated with an adverse metabolic profile and suggest a novel unifying mechanism for the previously independent observations that low testosterone levels and impaired mitochondrial function promote insulin resistance in men.\textsuperscript{35}

Another study showed that, in older men, lower TT is associated with insulin resistance independently of measures of central obesity. This association is seen with testosterone levels in the low to normal range.\textsuperscript{36} The increased incidence of diabetes was also confined to the lowest quintile of SHBG values.\textsuperscript{37} SHBG has been generally found to be negatively correlated with fasting insulin levels.\textsuperscript{38} Whether SHBG is a marker of insulin secretion and or insulin resistance has been debated.\textsuperscript{39} Sensitivity of FT assay was $0.005$ nmol/L. The interassay CVs were of $8.8\%$ and $7.9\%$ at FT concentrations of $0.018$ nmol/L and $0.85$ nmol/L, while the intraassay CVs were of $7.4\%$ and $7.1\%$ at the same FT concentrations, respectively. Referring to the normal values used by Baisley et al.,\textsuperscript{40} FT levels in normal SHBG phenotype and variant SHBG phenotype in this study [Table 3] were within the range in normal values. FT levels between normal SHBG phenotype when compared with variant SHBG phenotype did not differ ($p = 0.778$). Result of another research showed that plasma SHBG concentration was related to nonSHBG-bound testosterone.\textsuperscript{27} Some studies

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<th>Table 1: Characteristics of subjects</th>
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<td>Height (m)</td>
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<td>Body mass index (kg/m(^2))</td>
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<td>Ratio of protein–lipid</td>
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<th>Table 2: Molecular weight estimate of LMW and standard of SHBG and serum SHBG samples</th>
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<td>Proteins</td>
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<td>LMW standard</td>
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<td>Serum albumin</td>
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<td>Ovalbumin</td>
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<td>Carbonat anhidrase</td>
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<td>Trypsin inhibitor</td>
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<td>SHBG standard</td>
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<td>Normal SHBG phenotype (two bands)</td>
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<td>Normal SHBG phenotype (three bands)</td>
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<td>Variant SHBG phenotype (three bands)</td>
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LMW, low molecular weight; SHBG, sex hormone-binding globulin; MW, molecular weight; $R_f$, relative mobility; kDa, kilodalton.

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<th>Table 3: Comparison of SHBG, testosterone, and insulin levels between normal SHBG and variant SHBG phenotype groups</th>
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<tr>
<td>Variables</td>
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<tr>
<td>SHBG (nmol/L)</td>
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<td>TT (nmol/L)</td>
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<td>FT (nmol/L)</td>
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<td>Insulin (µIU/mL)</td>
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SHBG, sex hormone-binding globulin; TT, total testosterone; FT, free testosterone; nmol/L, nano mol per liter; SD, standard of deviation; sig., significance.
show that the relationship of FT and metabolic syndrome has been inconsistent or weak,\cite{30,31} suggesting that SHBG may be the primary determinant of the apparent relationship between TT and metabolic syndrome.\cite{20}

Sensitivity of insulin assay was 2.9 μIU/mL. The interassay CVs were of 9.7% and 7.1% at insulin concentrations of 5 μIU/mL and 100 μIU/mL, while the intraassay CVs were of 8.2% and 4.7% at the same insulin concentrations, respectively. The mean level of insulin in this study was 6.7 uIU/mL [Table 3], which was equal with the results of the study on 324 Korean men.\cite{41} Serum insulin level between normal SHBG phenotype when compared with variant SHBG phenotype was different ($p < 0.05$) [Table 3]. These data indicate that serum insulin level is influenced by proteins polymorphism of SHBG. Serum insulin levels in variant SHBG phenotype are high than normal SHBG phenotype, probably that more glycosylation is found in subjects with variant SHBG phenotype when compared with normal SHBG phenotype; so, we suspect that insulin is needed in the process of glycosylation.

We know that there is a relationship of glucose utilization by cells with insulin availability. Glucose transporters (GLUTs) 2 mediate ATP-independent facilitative diffusion of glucose across cell membranes.\cite{42} The facilitative glucose transporter GLUT4 plays a key role in regulating whole body glucose homeostasis. GLUT4 dramatically changes its distribution upon insulin stimulation, and insulin-resistant diabetes is often linked with compromised translocation of GLUT4 under insulin stimulation. Result of a study showed that NG is important for insulin-mediated cell surface. The NG chain is important in quality control and intracellular trafficking of GLUT4.\cite{43}

On the basis of the fact that the levels of SHBG, TT, and FT between normal SHBG phenotype do not differ with variant SHBG phenotype ($p > 0.05$); however, the insulin levels between normal SHBG phenotype are different from variant SHBG phenotype ($p < 0.05$). We conclude that, in adult healthy men, protein polymorphisms of SHBG was not found to affect the levels of SHBG and testosterone. Protein polymorphism of SHBG was found to affect insulin levels in the blood serum.

**Conclusion**

Protein polymorphisms of SHBG was not found to affect the levels of SHBG, TT, and FT. Protein polymorphism of SHBG was found to affect insulin levels in the blood serum.

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